Effect of specific phospholipid molecular species incorporated in human platelet membranes on thromboxane A_2/prostaglandin H_2 receptors

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Abstract The incorporation of albumin-bound docosahexaenoic acid (22:6n-3), but not linoleic acid (18:2n-6), into cellular phospholipids inhibits platelet aggregation induced by the thromboxane analogue U46619. [^{3}H]U46619 specific binding to thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptors, as well as specific binding of the antagonist [^{3}H]SQ29548 to these sites were also decreased in these modified cells (P. G., Swann et al. 1990. *J. Biol. Chem.* **265:** 21692–21697). More than 80% of the 22:6n-3 incorporated in these cells was esterified in the various endogenous phospholipid classes and the remaining was found in neutral lipids and in the unesterified fatty acid pool. In this study, we determined whether the effects observed could be attributed to the esterification of 22:6n-3 in phospholipids and whether the 22:6n-3 biological activity might depend on its esterification in specific phospholipid classes. Therefore, pure phosphatidylcholine (PC) and phosphatidylethanolamine (PE) molecular species were transferred to platelet membranes, using lipid transfer proteins. PC and PE containing palmitate (16:0) and 22:6n-3 or 16:0 and 18:2n-6 at position *sn*-1 and *sn*-2, respectively, were incorporated into membranes only at the expense of the corresponding endogenous phospholipid class, by an apparent exchange process. When such modified membranes were tested for specific binding of U46619 and SQ29548, a significant decrease of the receptor site affinity was only observed in membranes highly enriched with 1-palmitoyl-2-docosahexaenoyl-glycerophosphocholine (16:0/22:6-GPC). Fluidity parameters measured by electron spin resonance of 5- and 16-nitroxy-stearic acids were not significantly different in membranes enriched with 16:0/22:6-GPC relative to those enriched with 16:0/18:2n-6-GPC, arguing against a generalized perturbation of the membrane due to 22:6n-3 incorporation. We conclude that molecular species of PC with 22:6n-3 at the *sn*-2 position can affect TXA_2/PGH_2 receptors. The selectivity of the inhibitory effect of PC containing 22:6n-3 is discussed.—**Bayon, Y., M. Croset, D. Daveloose, F. Guerbette, V. Chirouze, J. Viret, J-C. Kader, and M. Lagarde.** Effect of specific phospholipid molecular species incorporated in human platelet membranes on thromboxane A_2/prostaglandin H_2 receptors. *J. Lipid Res.* 1995. **36:** 47–56.

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Eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids are the main polyunsaturated fatty acids of fish oil that have been found able to decrease the atherothrombotic risk in humans (1, 2). This potential has been attributed to 20:5n-3, as a structural analogue of arachidonic acid (20:4n-6), in competing with the cellular metabolism of the latter at both the phospholipid and the oxygenase levels. Specifically, 20:5n-3 competes in the formation of thromboxane A_2 (TXA_2), a potent inducer of blood platelet aggregation and a potent vasoconstricting agent, and forms thromboxane A_3, a prostanoid much less active than TXA_2 (3–5). However, 22:6n-3 may also contribute to inhibit platelet aggregation by fish oil with a mechanism of action substantially different from that of 20:5n-3 as it is poorly liberated from membrane phospholipids by phospholipase A_2 activities and is not metabolized by the platelet prostaglandin H synthase (6). This suggests that biological activities of 22:6n-3 are physiologically relevant only when they are observed with the fatty acid esterified in phospholipids. Unesterified 22:6n-3 and 20:5n-3, to a lesser extent, competitively and selectively inhibit U46619-induced platelet aggregation as well as [^{3}H]U46619 specific binding to TXA_2/prostaglandin H_2 (PGH_2) receptors (7). In vitro incorporation of 22:6n-3 and 20:5n-3 into the different pools of platelet lipids also led to inhibition of U46619-induced platelet aggregation and to decreased U46619 affinity for its receptor. Even though 22:6n-3 and 20:5n-3 incorporated into

Abbreviations: B_{max}, maximal binding capacity; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; K_d, dissociation constant; K_i, inhibitory constant; LTP, lipid transfer protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PGH_2, prostaglandin H_2; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; TXA_2, thromboxane A_2; GLC, gas-liquid chromatography.

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platelets were esterified mostly in phospholipids, the inhibitory effects of these fatty acids on TXA_2/PGH_2 receptors might be partly explained by enrichment of the other lipid fractions in 22:6n-3 and 20:5n-3. Indeed, 5-15% of these fatty acids were found esterified in neutral lipids and about 5% remained in the unesterified form. When incorporated in platelet lipids, 22:6n-3 and 20:5n-3 can selectively modulate the TXA_2/PGH_2 receptor function as they do not affect TXA_2/PGH_2-independent platelet aggregation induced by thrombin or A23187 (8). Neither 22:6n-3 nor 20:5n-3 in unesterified or esterified forms altered epinephrine-induced aggregation or [^{3}H]yohimbine binding to platelet α_2-adrenergic receptors (9). Among all the fatty acids tested, including linoleic acid (18:2n-6), only 22:6n-3 and 20:5n-3 inhibited specifically TXA_2/PGH_2 receptors of whole platelets (8) or of solubilized platelet membranes (9), and 22:6n-3 was generally much more potent than 20:5n-3. These findings support the notion that 22:6n-3 and 20:5n-3 can selectively and directly interact with TXA_2/PGH_2 receptors.

Based on these considerations, the present study was designed to determine whether 22:6n-3 esterified in the major platelet phospholipid classes could influence the binding of TXA_2/PGH_2 mimetics to TXA_2/PGH_2 receptors of human platelet membranes and whether the effect observed depends on specific phospholipid classes containing 22:6n-3. Using lipid transfer proteins (LTP), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) with 18:2n-6 and 22:6n-3 at the *sn*-2 position were then incorporated into platelet membranes by an apparent exchange process. It was found that the TXA_2/PGH_2 receptor activity was only inhibited by increasing 22:6n-3 proportion in PC, an effect that was not accompanied by a change of the membrane fluidity.

MATERIALS AND METHODS

Materials

Phosphatidylinositol (PI) from bovine liver, 1-palmitoyl-2-linoleoyl-glycerophosphocholine (16:0/18:2-GPC) and 1-palmitoyl-2-linoleoyl-glycerophosphoethanolamine (16:0/18:2-GPE) were obtained from Sigma (St. Louis, MO). 1-Palmitoyl-2-docosahexaenoyl-GPC (16:0/22:6-GPC) and 1-palmitoyl-2-docosahexaenoyl-GPE (16:0/22:6-GPE) were supplied by Avanti Polar Lipids (Alabaster, AL). U46619 was from Cascade Biochem Ltd. (Reading, England). [^{3}H]glycerol trioleate (26.8 Ci/mmol, 991.6 GBq/mmol) and 5,6-[^{3}H]SQ29548 (58.00 Ci/mmol, 1.85 Gbq/mmol) were purchased from DuPont New England Nuclear (Boston, MA). SQ29548 was a gift from Dr. M. Ogletree (Squibb Institute for Research, Princeton, NJ). The maize lipid transfer protein was prepared as previously described (10). Stearic acids with the 4-4' dimethyl-3-oxazolidinyloxy group attached to either carbon atom 5 (5-nitroxy stearic acid) or 16 (16-nitroxy stearic acid) were from Molecular Probes (Eugene, OR). All the other reagents were of analytical grade (Merck, Darmstadt, Germany, Sigma, St. Louis, MO or Rhône Poulenc, Peypin, France).

Incorporation of phospholipids into platelet membranes

Preparation of platelet membranes. Platelets were prepared as previously reported (11) and platelet membranes were obtained by mild sonication of washed platelets. Briefly, blood from healthy volunteers was collected into 1/9 vol of anticoagulant (citric acid/citrate/dextrose, pH 4.5) and platelet-rich plasma (PRP) was prepared by centrifugation at 180 *g* for 18 min. PRP (pH 6.4) was centrifuged at 900 *g* for 12 min and the resulting platelet pellet was gently resuspended in a sucrose/Tris-HCl buffer (100 μM phenylmethanesulphonyl fluoride, 2 μM 2-4'-dibromoacetophenone, 1 mM EDTA, 280 mM sucrose, 5 mM Tris-HCl, pH 7.4). The platelet suspension (3×10^9 platelets/ml) was sonicated at setting 2 for 15 sec at 4°C using a Branson sonicator with a small tip. The sonicate was centrifuged at 1200 *g* for 10 min at 4°C, the supernatant was retained, and the pellet of unbroken cells and debris was resuspended into the sucrose/Tris-HCl buffer and re-sonicated. This last procedure was repeated twice. After the sonication step, the three supernatants were combined and centrifuged at 13,000 *g* for 20 min at 4°C. The resulting supernatant constituted the platelet homogenate which was used for the enrichment experiments.

Preparation of platelet phospholipids deprived of phosphatidylethanolamine. Lipids were extracted from about 10^{11} platelets according to the Bligh and Dyer procedure (12). Phospholipids (PC, phosphatidylserine [PS], PI, and sphingomyelin) were then purified by thin-layer chromatography on semi-preparative silica gel H plates developed with chloroform-methanol-40% aqueous methylamine 60:20:5 (v/v/v) (13). They were quantified by phosphorus determination according to the method of Rouser, Fleisher, and Yamamoto (14).

Preparation of vesicles. Small phospholipid unilamellar vesicles were obtained by resuspending the lipids in 5 mM Tris-HCl buffer, pH 7.4. The lipid dispersion was sonicated at setting 1 for 12 min under nitrogen in a Branson sonicator using a small titanium tip and centrifuged for 20 min at 45,000 *g* to remove metallic particles and large aggregates. Recovery of the lipids in the supernatant was higher than 90%. Under these conditions, phospholipids were found to be unaltered.

Enrichment of platelet membrane diacylglycerophosphocholine (diacyl-GPC) and diacyl-glycerophosphoethanolamine (diacyl-GPE) in linoleic and docosahexaenoic acids. Diacyl-GPC in platelet membranes were enriched in linoleic and docosahexaenoic acids using the endogenous PI/PC-transfer protein (15). The platelet homogenate (7.5 mg protein) was incubated for 30 min at 30°C with unilamellar vesi-

cles containing 85 or 335 nmol of phospholipids consisting of 10 mol of PC (16:0/18:2-GPC or 16:0/22:6-GPC) for 1 mol of bovine liver PI. Preliminary experiments showed that the transfer rate of diacyl-GPC was maximal during the first 30 min. For the diacyl-GPE enrichment assays, the platelet homogenate (7.5 mg protein) was incubated for 30 min at 30°C with 75 μg lipid transfer protein and unilamellar vesicles containing 150 or 300 nmol of phospholipids. These donor vesicles were made up of 1 mol of PE (16:0/18:2-GPE or 16:0/22:6-GPE) for 3 mol of platelet phospholipids. Control incubations contained the platelet homogenate without vesicles. For transfer assays, the extent of phospholipid spontaneous transfer was controlled by adding trace amounts of trioleoyl [^{3}H]glycerol, a nonexchangeable marker, into the vesicles. Preliminary experiments showed clearly that the measured nonspecific transfer of phospholipids was the same when trioleoyl [^{3}H]glycerol was applied alone or with the radiolabeled phospholipids in small unilamellar vesicles. The nonspecific transfer of phospholipids defined as the nonspecific sticking of small unilamellar vesicles to platelet membranes represented less than 25% of the total transferred 16:0/18:2- and 16:0/22:6-GPC.

After phospholipid transfers, the platelet homogenate was centrifuged at 105,000 *g* for 100 min, and the pellet was resuspended into Tris-HCl/$MgCl_2$ buffer (5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4).

Phospholipid analysis

Lipids were extracted from platelet membranes according to the procedure of Bligh and Dyer (12). Individual phospholipids were resolved by one dimensional thin-layer chromatography on silica gel 60G plates with chloroform–methanol 8:1 (v/v) as the predeveloping solvent and chloroform–methanol–40% aqueous methylamine 60:25:5 (v/v/v) as the developing solvent.

PC and PE subclasses were separated as described before except for slight modifications (16). Briefly, phospholipid classes were hydrolyzed into their respective diradylglycerols by incubation with phospholipase C according to Takamura et al. (17). Diradylglycerols were then converted to their benzoyl derivatives by treatment with dimethylaminopyridine and benzoic anhydride in dry benzene for 60 min at 20–25°C. The subclasses of diradylglycerobenzoates were further resolved by thin-layer chromatography on silica gel 60G plates using a solvent system of toluene–hexane–diethylether 50:45:4, (v/v/v) (18).

The fatty acid composition of phospholipids in control and modified platelet membranes was analyzed by gas–liquid chromatography. Phospholipids, except sphingomyelin, were treated with 14% BF_3 in methanol (19). Sphingomyelin fatty acids were treated with 5% H_2SO_4 in methanol at 95°C for 180 min. The resulting fatty acid methyl esters and dimethylacetals were analyzed using a Delsi chromatograph model DI 200, equipped with an SP 2380 capillary column (30 m × 0.25 mm) (Supelco, Bellefonte, PA). Phospholipids were quantified by gas–liquid chromatography using internal standards (17:0/17:0-GPC and 17:0/17:0-GPE).

Radioligand binding studies

Saturation binding experiments were performed with platelet membranes using the tritiated PGH_2/TXA_2 receptor antagonist, 5,6-[^{3}H]SQ29548 as described previously, with minor modifications (9, 20). The incubation mixture (1 ml final volume, Tris/$MgCl_2$ buffer) consisted of 60–75 μg platelet membrane proteins, 0.5 nM 5,6-[^{3}H]SQ29548, and increasing concentrations of SQ29548 (6–9 concentrations in duplicate from 0.5 to 100 nM). Assays were performed at 25°C for 30 min, then 4 ml of ice-cold assay buffer was added. The mixture was then rapidly filtered through Whatman GF/C fiber filters followed by two additional washings with 4 ml of the ice-cold buffer. The filtration procedure was complete within 10 sec. The filters were counted in a Packard Scintillation counter. Specific binding was defined as the total binding minus binding activity that could not be competed for by 10 μM SQ29548 and represented typically 85% of the total radioactivity bound. The maximal binding capacity (B_{max}) and the dissociation constant (K_d) were determined by Scatchard analysis using the EBDA program (21).

For competition binding experiments, 5,6-[^{3}H]SQ29548 (0.75 nM) was incubated with platelet membranes in the presence of varying concentrations of the unlabeled PGH_2/TXA_2 receptor agonist, U46619 (6–9 concentrations in duplicate from 20 to 1000 nM), for 30 min at 25°C. Nonspecific binding was defined as the amount of radioactivity bound in the presence of unlabeled SQ29548 (10 μM). The inhibitory constant (K_i) and the slope, an indirectly determined Hill coefficient, were obtained from the analysis of competition binding assays with the EBDA program (21).

Electron spin resonance experiments

Electron spin resonance experiments were performed with a spectrometer E-109 X-Band Varian equipped with a nitrogen gas flux temperature regulation. The labeling of membranes was carried out by addition of the spin label in dimethyl sulfoxide, at 10^{-2} M for the 5-nitroxy stearic acid spin probe and 2.5×10^{-3} M for the 16-nitroxy stearic acid spin probe, to obtain a final concentration of 2×10^{-4} M and 5×10^{-5} M, respectively, with the critical condition of less than 2% (v/v) of dimethyl sulfoxide.

Spectra were recorded between 2 and 40°C; the temperature was regulated with an accuracy of ± 0.1°C. Then, each spectrum was digitized with an HP9874A, and spectral parameters were calculated with the HP9825T calculator.

The values of the order parameter "S" obtained with the 5-nitroxy stearic acid spin probe were calculated for each temperature with the following relation:

$$S = \frac{2A_{//mes} - 2A_{\perp mes}}{2A_{//th} / 2A_{\perp th}}$$

where $2A_{//mes}$ and $2A_{\perp mes}$ are the maximum and minimum hyperfine splitting constants measured on the spectra, respectively, and $2A_{//th}$ and $2A_{\perp th}$ are the theoretical values determined for a single oriented crystal (22). Then, we plotted the variation of the order parameter S versus temperature and determined the regression line which allows us to calculate this parameter with a good accuracy, at 25°C.

For the 16-nitroxy stearic acid spin probe, the spectra were analyzed in terms of rotational correlation time τ_c obtained from the equation of Henry and Keith (23):

$$\tau_c = 6.65 \times 10^{-10} \times W_0 \times (\sqrt{H_0/H_{+1}} - 1)$$

where W_0 and H_0 are the line width and the amplitude of the central line of the spectra, respectively. We proceeded as for the order parameter since τ_c was calculated at each temperature. The calculation of the regression line allowed to determine the rotational correlation time at 25°C with a good accuracy.

Statistical analysis

One-way analyses of variance were performed to determine differences at various levels of significance. This was followed by the Fisher's PLSD post hoc test to determine differences between groups. The Student's *t* test was also used.

RESULTS

Enrichment of platelet membrane diacyl-GPC in 18:2n-6 and 22:6n-3

As shown in **Fig. 1,** the activity of the endogenous platelet PC/PI-transfer protein was found high enough to promote the enrichment of platelet membrane diacyl-GPC in 18:2n-6 and 22:6n-3 by transferring 16:0/18:2-GPC and 16:0/22:6-GPC, respectively. The endogenous levels of 18:2n-6 and 22:6n-3 in control platelet diacyl-GPC were 10.7 ± 0.9 and 0.8 ± 0.1 mol % of the total PC fatty acids, respectively. After the transfer of two different amounts of 16:0/18:2-GPC and 16:0/22:6-GPC, the 18:2n-6 proportion was increased to 13.4 ± 0.3 and 17.4 ± 0.8 mol % and that of 22:6n-3 to 5.8 ± 0.7 and 10.3 ± 0.9 mol %. The elevation of 18:2n-6 proportion in diacyl-GPC was mainly counterbalanced by a decrease of oleic acid (18:1n-9) and 20:4n-6. Similarly, the 22:6n-3 proportion increased at the expense of that of 18:1n-9, 18:2n-6 and 20:4n-6 altogether (Fig. 1).

The selectivity of this enrichment process was assessed by analyzing PC, PE, and PI after enrichment of platelet membrane diacyl-GPC in 18:2n-6 and 22:6n-3. The fatty acid composition changed markedly only in PC after transfer of 16:0/18:2- or 16:0/22:6-GPC whereas it was not modified in PE and tended to be slightly but not significantly altered in PI (data not shown).

To ascertain that the experimental approach resulted essentially in exchange of platelet membrane phosphatidylcholine, phospholipid classes were quantified by GLC using internal standards (**Table 1**). The relative amounts of PC, PE, and PI in modified membranes did not differ notably from that observed in control membranes. However, 10% increase of PC content was observed in platelet membranes incubated with the higher concentration of vesicles containing 1-palmitoyl-2-docosahexaenoyl-GPC (Table 1).

Enrichment of platelet membrane diacyl-GPE in 18:2n-6 and 22:6n-3

In the same way, the 18:2n-6 and 22:6n-3 proportions in diacyl-GPE were modified after the maize lipid transfer protein-catalyzed transfer of 16:0/18:2-GPE and 16:0/22:6-GPE, respectively, to platelet membranes (**Fig. 2**). The initial proportions of 18:2n-6 and 22:6n-3 in platelet PE were 6.0 ± 0.2 and 2.2 ± 0.9 mol % of the total PE fatty acids, respectively. After lipid transfer protein-catalyzed enrichment, 18:2n-6 proportions reached 17.1 ± 0.6 and 21.2 ± 0.5 mol %, and 22:6n-3 levels increased to 12.5 ± 0.4 and 17.2 ± 0.5 mol %. The incorporation of 18:2n-6 and 22:6n-3 at the *sn*-2 position of diacyl-GPE induced decreased proportions of 18:1n-9 and 20:4n-6. In addition, the enrichment process induced modifications in diacyl-GPE proportions of stearic acid (18:0) and palmitic acid (16:0), fatty acids found principally at the *sn*-1 position. Marked enhancement (2.5-fold) of the 16:0 proportion, which reached 23 mol % of total diacyl-GPE fatty acids, was noted while that of 18:0 decreased concomitantly to 21 mol % (1.5-fold reduction) (Fig. 2). When comparing enriched platelet membranes by one-way analysis of variance, only 18:2n-6 and 22:6n-3 proportions in PE were significantly different after the transfer of the same amount of 16:0/18:2- or 16:0/22:6-GPE to membranes.

The diacyl-GPE enrichment process was highly selective for the phospholipid class. After incorporation of 16:0/18:2- or 16:0/22:6-GPE in platelet membranes, the fatty acid composition was altered in PE, whereas it was unchanged in the other major platelet phospholipid classes (data not shown). A slight decrease (5-10%) of the relative amounts of PC, PE, PS, and PI occurred in modified platelet membranes as compared to controls (**Table 2**).

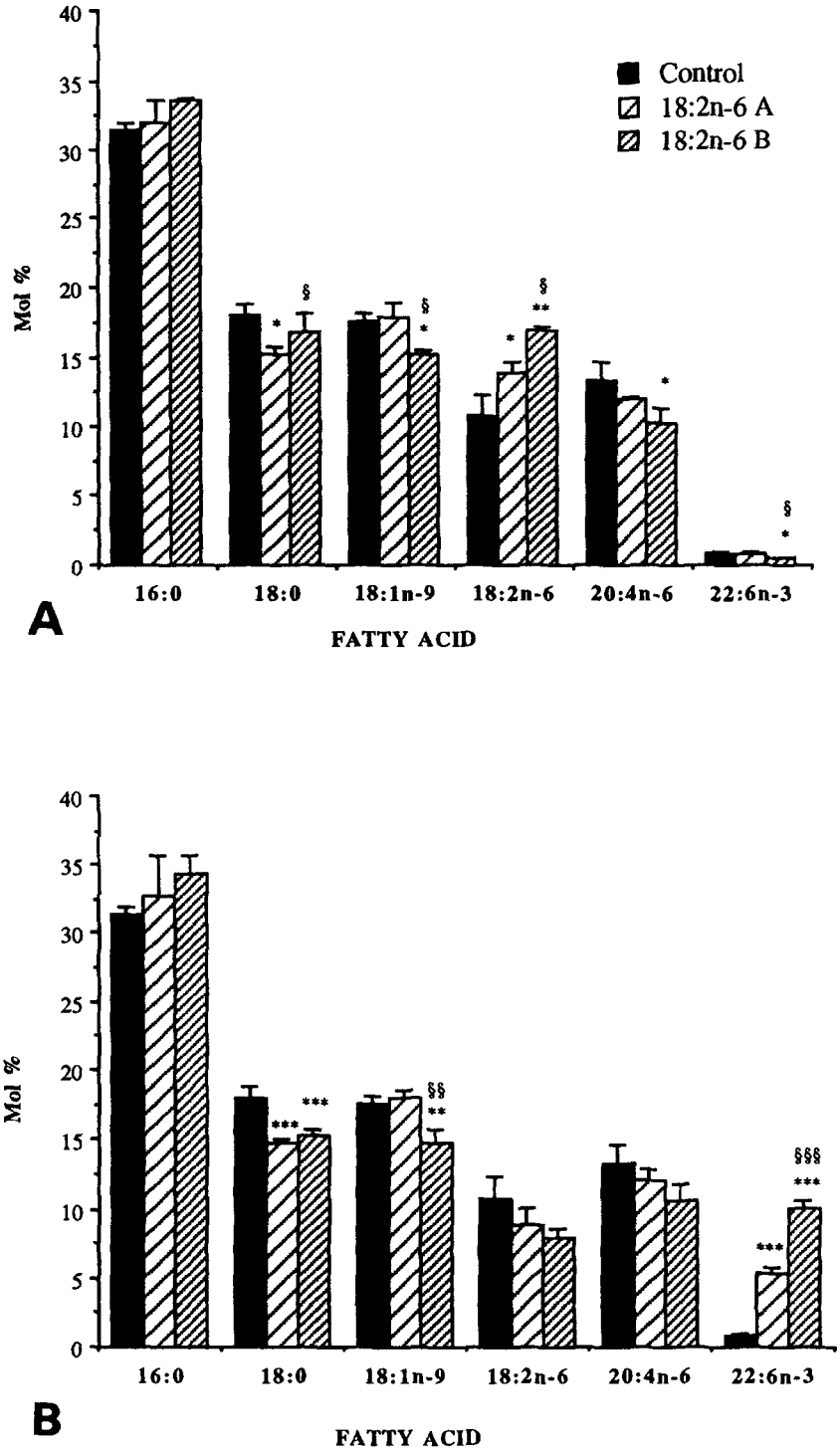

Fig. 1. Fatty acid composition of diacyl-GPC from platelet membranes after enrichment with 16:0/18:2-GPC or 16:0/22:6-GPC. Diacyl-GPC of platelet membranes were enriched with either 16:0/18:2-GPC (18:2n-6 A, 18:2n-6 B) (Fig. 1A) or 16:0/22:6-GPC (22:6n-3 A, 22:6n-3 B) (Fig. 1B) using the platelet PI/PC-transfer protein as described in Materials and Methods. Assays designated by A (18:2n-6 A, 22:6n-3 A) and B (18:2n-6 B, 22:6n-3 B) represent the low and the high enrichment levels of diacyl-GPC with the fatty acids, respectively. Donor phospholipid vesicles contained 85 nmol (A) or 335 nmol (B) of phospholipids. Donor phospholipid vesicles were made up of diacyl-GPC/bovine liver PI, 10/1 mol/mol. Control platelet membranes (black bar) were obtained by incubation without vesicles. Results on fatty acid composition are expressed in mol % of total fatty acids and data are means ± SD of three experiments. Statistical significance (ANOVA followed by Fisher's PLSD post hoc test) of modified versus control membranes and of the lower versus the higher enriched membranes in 18:2n-6 or 22:6n-3 are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and by $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$, respectively.

TABLE 1. PC, PE, and PI contents of platelet membranes after transfer of 16:0/18:2-GPC and 16:0/22:6-GPC

	Relative Quantity (%)		
	PC	PE	PI
Control	100	100	100
18:2n-6 A	105.6 ± 5.4	106.0 ± 4.7	105.4 ± 2.8
18:2n-6 B	100.8 ± 6.6	96.3 ± 12.1	95.3 ± 1.9
22:6n-3 A	99.5 ± 4.9	95.9 ± 2.7	94.9 ± 6.8
22:6n-3 B	110.3 ± 2.3[a]	95.8 ± 4.0	98.4 ± 1.9

Phospholipid transfer conditions are given in Materials and Methods. PC and PI, the only phospholipids exchangeable by the platelet PI/PC-transfer protein, and PE, an important platelet phospholipid class, were quantified by GLC using an internal standard. The amount of each analyzed phospholipid class from control platelet membranes was taken as 100%. Assays designated by A and B represent the low and high enrichment levels of diacyl-GPC with the fatty acids, respectively. Data are means ± SD of three experiments.

[a] $P < 0.02$ when compared to control (Student's t test).

Effects of platelet membrane diacyl-GPC and -GPE enrichments with 18:2n-6 and 22:6n-3 on the [^{3}H]SQ29548 and U46619 binding to TXA_2/PGH_2 receptors

The effect of 22:6n-3 esterified in phospholipids on TXA_2/PGH_2 receptors was investigated in platelet membranes whose diacyl-GPC and -GPE were enriched selectively with 18:2n-6 or 22:6n-3. **Table 3** shows the effects of phospholipid enrichment in 18:2n-6 and 22:6n-3 on [^{3}H]SQ29548 binding parameters. For control membranes, values of the dissociation constant (K_d) and the maximal binding capacity of [^{3}H]SQ29548 are close to those reported previously (20). Alteration of the [^{3}H]SQ29548 binding was observed when the proportion of 22:6n-3 in diacyl-GPC was increased to the highest level. The corresponding enrichment induced a 65% increase of the K_d relative to control whereas the lower enrichment did not inhibit [^{3}H]SQ29548 specific binding. No changes in B_{max} relative to control were observed, and [^{3}H]SQ29548 apparently labeled a single class of receptors, whatever the molecular species transferred to membranes.

The effects of diacyl-GPC and -GPE enrichment in 18:2n-6 and 22:6n-3 on U46619 binding to TXA_2/PGH_2 receptors were also investigated. Consistent with the inhibition of [^{3}H]SQ29548 binding, the higher enrichment of diacyl-GPC with 22:6n-3 caused an alteration (a 1.5-fold increase) of the U46619 inhibitory constant (K_i) relative to control (**Table 4**). On the other hand, it did not induce changes of the slope. Again, no effect of membrane enrichment with either diacyl-GPC containing 18:2n-6 or diacyl-GPE containing 18:2n-6 or 22:6n-3 could be observed. In every case, slopes were close to 1, suggesting that U46619 apparently failed to discriminate between different subtypes of receptors in our conditions.

Influence of platelet membrane diacyl-GPC enrichment with 18:2n-6 and 22:6n-3 on fluidity parameters

Fluidity parameters acquired with electron spin resonance probes were determined from platelet membranes with diacyl-GPC enriched with 22:6n-3. They were compared to control membranes and membranes obtained after the higher diacyl-GPC enrichment with 18:2n-6 (**Table 5**). Diacyl-GPC enrichment with either 18:2n-6 or 22:6n-3 had no marked effect on fluidity parameters. The order parameter and the rotational correlation time tended to decrease and increase, respectively, in modified membranes when compared to control. However, these changes were not statistically significant, and both fluidity parameters were identical in membranes after the highest diacyl-GPC enrichment with either 18:2n-6 or 22:6n-3.

DISCUSSION

It has been shown that 22:6n-3 is a potent inhibitor of TXA_2 action in decreasing the human platelet TXA_2/PGH_2 receptor affinity for its ligands when most of 22:6n-3 was esterified in membrane phospholipids (8). The 22:6n-3 inhibitory effect appeared relatively specific for this receptor type as 22:6n-3 failed to alter human platelet α_2-adrenergic receptors (9) which present a relevant homologous structure with human TXA_2/PGH_2 receptors (24). Diets enriched in 22:6n-3 have also been found to inhibit ex vivo platelet aggregability in response to U46619 in humans (25). However, diets containing high levels of n-3 polyunsaturated fatty acids fed to rats did not modify the affinity of the TXA_2/PGH_2 receptors for SQ29548 and U46619 and seemed to stimulate platelet functions induced by U46619 by increasing the efficiency of the thromboxane signal transduction (26).

The present experiments extend these studies by specifically enriching platelet membrane diacyl-GPC and -GPE, the two main platelet phospholipid subclasses, with 22:6n-3 and 18:2n-6. The phospholipid enrichment with 18:2n-6 and 22:6n-3 was obtained by transferring pure molecular species (16:0/18:2-, 16:0/22:6-GPC, 16:0/18:2-, or 16:0/22:6-GPE) to membranes. This enrichment process was achieved by using the platelet PI/PC transfer protein and the maize lipid transfer protein in conditions allowing an exchange rather than a net transfer of phospholipids (Tables 1 and 2). The incorporated PUFA were not redistributed in other phospholipid classes (data not shown). This was a major advantage of using platelet membranes instead of whole cells where an active remodelling of phospholipids occurs (27).

The TXA_2/PGH_2 receptors were not altered by our conditions of platelet membrane preparation. The binding parameters of SQ29548 obtained for control mem-

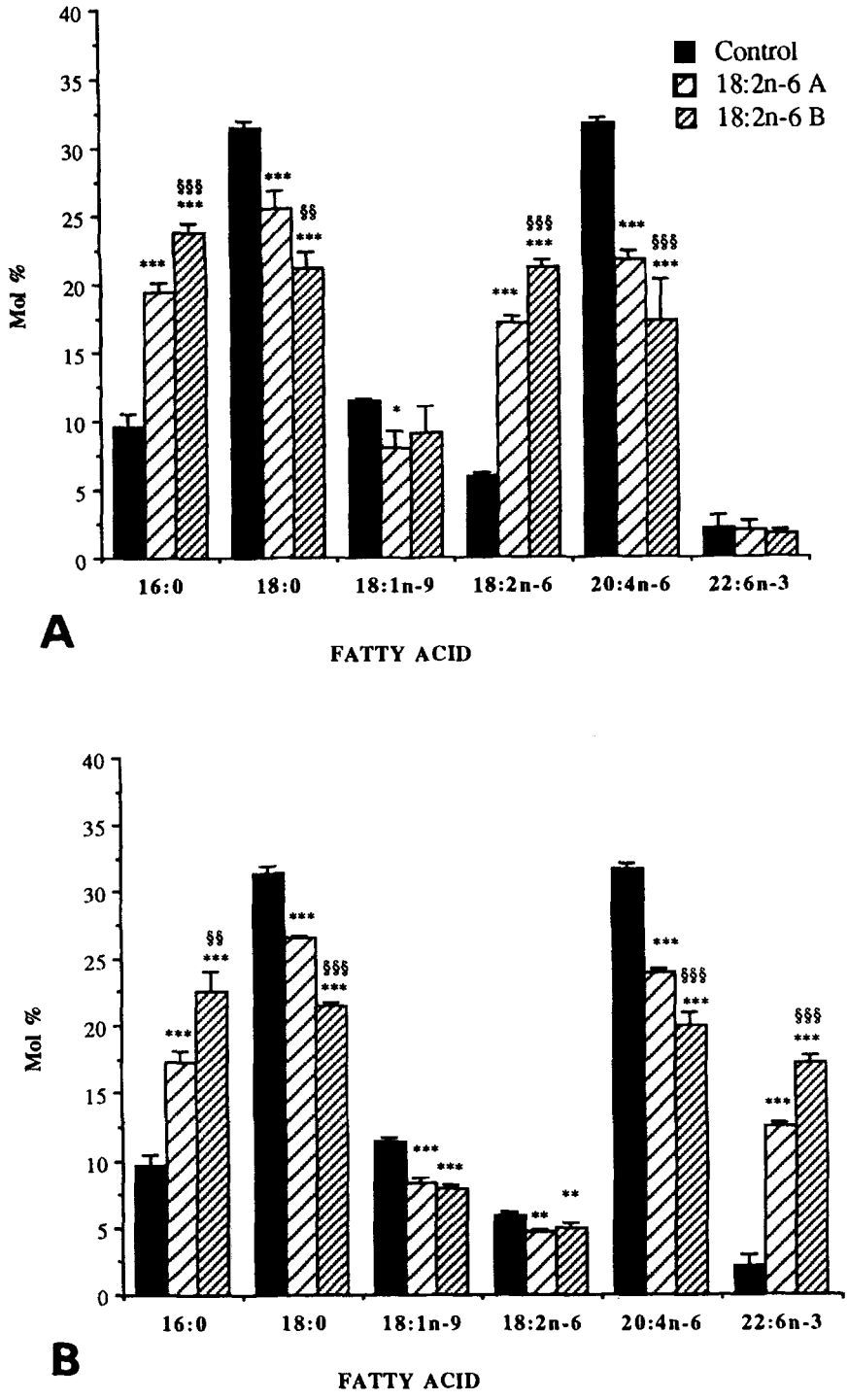

Fig. 2. Fatty acid composition of diacyl-GPE from platelet membranes after enrichment with 16:0/18:2-GPE or 16:0/22:6-GPE. Diacyl-GPE of platelet membranes were enriched with either 16:0/18:2-GPE (18:2n-6 A, 18:2n-6 B) (Fig. 2A) or 16:0/22:6-GPE (22:6n-3 A, 22:6n-3 B) (Fig. 2B) using the maize lipid-transfer protein as described in Material and Methods. Assays designated by A (18:2n-6 A, 22:6n-3 A) and B (18:2n-6 B, 22:6n-3 B) represent the low and the high enrichment levels of diacyl-GPE with the fatty acids, respectively. Donor phospholipid vesicles contained 150 (A) nmol or 300 nmol (B) of phospholipids. Donor phospholipid vesicles were made up of diacyl-GPE/platelet phospholipids, 1/3 mol/mol. Control platelet membranes (black bar) were obtained by incubation without vesicles and without the maize lipid-transfer protein. Results on fatty acid composition are expressed in mol % of total fatty acids and data are means ± SD of three experiments. Statistical significance (ANOVA followed by Fisher's PLSD post hoc test) of modified versus control membranes and of the lower versus the higher enriched membranes in 18:2n-6 or 22:6n-3 are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and by $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$, respectively.

TABLE 2. PE, PC, PS, PI, and sphingomyelin contents of platelet membranes after transfer of 16:0/18:2-GPE and 16:0/22:6-GPE

	Relative Quantity (%)				
	PE	PC	PS	PI	Sphingomyelin
Control	100	100	100	100	100
18:2n-6 A	93.0 ± 8.0	92.8 ± 9.2	87.5 ± 3.3[a]	91.3 ± 1.9[b]	102.4 ± 4.2
18:2n-6 B	90.3 ± 4.5	94.6 ± 7.8	89.7 ± 5.4	89.0 ± 3.6[a]	100.9 ± 9.0
22:6n-3 A	97.6 ± 7.1	95.0 ± 9.7	93.9 ± 3.8	92.1 ± 4.8	101.9 ± 8.7
22:6n-3 B	94.2 ± 8.8	97.1 ± 6.1	95.5 ± 6.4	91.5 ± 5.7	100.9 ± 7.3

Phospholipid transfer conditions are given in Materials and Methods. Assays designated by A and B represent the low and high enrichment levels of diacyl-GPE with the fatty acids, respectively. PE, PC, PS, PI, and sphingomyelin, the main platelet phospholipid classes which can be exchanged by the maize lipid transfer protein, were quantified by GLC using an internal standard. The amount of each analyzed phospholipid class from control platelet membranes was taken as 100%. Data are means ± SD of three to four experiments.

[a] $P < 0.05$; [b] $P < 0.02$ when compared to control (Student's t test).

branes (K_d: 4.1 and 4.5 nM; B_{max}: 1900–2100 fmol/mg of protein) are in agreement with those reported previously for human washed platelets (K_d: 5.2 nM and B_{max}: 2580 fmol/mg of protein, data from ref. 20) and platelet membranes (K_d: 11.3 nM and B_{max}: 1466 fmol/mg of protein, data from ref. 20; B_{max}: 2206 fmol/mg of protein from Webb et al., unpublished data cited in ref. 28). [^{3}H]SQ29548 apparently recognized only one class of receptors in accordance to the earlier report (20). Furthermore, the K_i of U46619 found in control membranes (100–104 nM) agrees with the K_d estimated from the Scatchard analysis of [^{3}H]U46619 to intact platelets (132 nM) (8). However, we did not observe a biphasic inhibition of [^{3}H]SQ29548 binding to platelet membranes by U46619 as observed by Hedberg et al. (20).

TABLE 3. Effect of platelet diacyl-GPC and diacyl-GPE enrichments with 18:2n-6 and 22:6n-3 on the SQ29548 affinity for the TXA_2/PGH_2 receptor

	Diacyl-GPC		Diacyl-GPE	
	K_d	B_{max}	K_d	B_{max}
	nM	*pmol/mg protein*	*nM*	*pmol/mg protein*
Control	4.5 ± 1.03	1.9 ± 0.2 (7)	4.1 ± 0.6	2.1 ± 0.2 (6)
22:6n-3 A	4.3 ± 0.3	1.8 ± 0.1 (3)	4.1 ± 1.0	1.9 ± 0.2 (3)
22:6n-3 B	7.6 ± 1.5[a]	2.1 ± 0.3 (4)	4.6 ± 0.6	2.0 ± 0.2 (3)
18:2n-6 A	4.3 ± 0.6	1.9 ± 0.2 (3)	4.1 ± 0.9	2.1 ± 0.2 (3)
18:2n-6 B	4.3 ± 1.0	1.9 ± 0.1 (4)	4.3 ± 0.7	2.2 ± 0.2 (3)

Diacyl-GPC or -GPE of platelet membranes were enriched with either 18:2n-6 (18:2n-6 A, 18:2n-6 B) or 22:6n-3 (22:6n-3 A, 22:6n-3 B) as described in Materials and Methods. Assays designated by A (18:2n-6 A, 22:6n-3 A) and B (18:2n-6 B, 22:6n-3 B) represent the low and the high enrichment levels of diacyl-GPC or -GPE with the fatty acids, respectively. See Results for the enrichment levels. The parameters of SQ29548 binding to platelet membranes, the dissociation constant (K_d), and the maximum binding capacity (B_{max}) were determined by Scatchard analysis using the EBDA program as indicated in Materials and Methods. Data are presented as means ± SD for (n) experiments.

[a] $P < 0.01$ when compared to K_d values of control, 22:6n-3 A, 18:2n-6 A, 18:2n-6 B corresponding to the diacyl-GPC group (Fisher's PLSD post hoc test).

The affinity of the human platelet TXA_2/PGH_2 receptors for SQ29548 and U46619 appeared to be selectively reduced when 22:6n-3 was present in diacyl-GPC. However, the absence of effect of diacyl-GPE containing 22:6n-3 might be explained by the relative amount of 22:6n-3 PC and PE molecular species transferred to platelet membranes. The endogenous concentration of diacyl subclass of PC in platelet membranes is about 3-fold higher than the concentration of diacyl subclass of PE (29). Therefore, a higher amount of 16:0/22:6-GPC as compared to 16:0/22:6-GPE was incorporated in platelet membranes in order not to induce any modification of the endogenous quantities of diacyl-GPC and -GPE. A net threshold effect of 16:0/22:6-GPC was observed. Indeed, TXA_2/PGH_2 receptor binding activity was only reduced

TABLE 4. Effect of platelet diacyl-GPC and diacyl-GPE enrichments with 18:2n-6 and 22:6n-3 on the U46619 affinity for the TXA_2/PGH_2 receptor

	Diacyl-GPC		Diacyl-GPE	
	K_i[a]	Slope	K_i	Slope
	nM		*nM*	
Control	104 ± 20	1.0 ± 0.0 (4)	100 ± 16	0.9 ± 1.0 (3)
22:6n-3 A	99 ± 12	0.9 ± 0.1 (4)	103 ± 20	0.9 ± 0.0 (3)
22:6n-3 B	159 ± 16[b]	1.0 ± 0.1 (4)	117 ± 20	0.9 ± 0.2 (3)
18:2n-6 A	100 ± 17	0.9 ± 0.1 (4)	110 ± 19	0.9 ± 0.1 (3)
18:2n-6 B	99 ± 22	0.9 ± 0.1 (4)	121 ± 10	1.0 ± 0.1 (3)

Platelet membranes were enriched with 18:2n-6 and 22:6n-3 as described in Materials and Methods. The different assays of enrichment are given in the legend for Table 3. The binding parameters of U46619 to platelet membranes, the inhibitory constant (K_i), and the slope, an indirectly determined Hill coefficient, were obtained from the analysis of competition binding assays with the EBDA program as indicated in Materials and Methods. Data are presented as means ± SD for (n) experiments.

[a] Inhibitory constant.

[b] $P < 0.01$ when compared to K_d values of control, 22:6n-3 A, 18:2n-6 A, 18:2n-6 B corresponding to the diacyl-GPC group (Fisher's PLSD post hoc test).

TABLE 5. Order parameter (S) and rotational correlation time (τ_c) of platelet membranes after diacyl-GPC enrichment with 18:2n-6 and 22:6n-3, at 25°C

	S	τ_c (ns)
Control	0.618 ± 0.002 (4)	1.19 ± 0.17 (4)
22:6n-3 A	0.607 ± 0.010 (4)	1.36 ± 0.07 (4)
22:6n-3 B	0.610 ± 0.003 (4)	1.37 ± 0.05 (4)
18:2n-6	0.611 ± 0.007 (3)	1.37 ± 0.16 (4)

Diacyl-GPC of platelet membranes were enriched with either 18:2n-6 (18:2n-6 B) or 22:6n-3 (22:6n-3 A, 22:6n-3 B) as described in Materials and Methods. Assays designated by A (22:6n-3 A) and B (18:2n-6 B, 22:6n-3 B) represent the low and the high enrichment levels of diacyl-GPC with the fatty acids, respectively. The order parameter (S) and the rotational correlation time (τ_c) were obtained by labeling platelet membranes with 5- and 16-nitroxy stearic acids, respectively, as described in Materials and Methods. Data are presented as means ± SD for (n) experiments.

in a significant manner after the higher enrichment of diacyl-GPC in 22:6n-3. There was also such an effect for the inhibition of [^{3}H]U46619 binding with intact platelets incubated in presence of increasing 22:6n-3/albumin molar ratios (8). All the results of the present study are in agreement with the previous findings, although the 22:6n-3 inhibitory effect observed in the present study was 2- to 3-times lower (7-9). These observations might be explained by the different states of coupling of the platelet TXA_2/PGH_2 receptors with G-proteins as suggested by Webb et al. (28). This hypothesis is supported by the different ability of U46619 to inhibit the binding of [^{3}H]SQ29548 to whole platelets and platelet membranes (Table 4) (20).

After lipid transfer protein-catalyzed phospholipid enrichment, the proportion of 22:6n-3 in platelet membrane phospholipids (3-5 mol % in comparison with 1-1.5 mol % for control membranes) was comparable to or greater than the level observed previously in human whole platelets in vitro and ex vivo after enrichment in 22:6n-3. It can reach 8 mol % after incubation of platelets with albumin bound 22:6n-3 (8, 16) and 3 mol % after diets enriched in 22:6n-3 (30-32). Platelets preferentially incorporated fatty acids associated with albumin into phospholipids, but also into other lipid classes, including neutral lipids and free fatty acids (8). Although the proportion of 22:6n-3 in neutral lipids was considered to be too low to inhibit TXA_2/PGH_2 receptors by itself (8), it could contribute to amplifying the 22:6n-3 phospholipid inhibitory effect. The biological activity of 22:6n-3 might depend on its specific esterification into other phospholipid pools in the membrane since 22:6n-3 associated with albumin was also significantly incorporated in platelet alkylacyl-GPC and alkenylacyl-GPE (16). The effect might be also modulated by the location of 22:6n-3 in multiple lipid or phospholipid classes.

It is likely that 22:6n-3 esterified in phospholipids is more or less potent depending on its location in the membrane. In particular, the selective inhibition of solubilized or partially purified TXA_2/PGH_2 receptors by 22:6n-3 (9) suggests that this fatty acid may directly interact with the receptors. Unlike 22:6n-3 carried by albumin, the transfer of 22:6n-3-phospholipids by LTP may not enrich both leaflets of platelet membrane vesicles. Indeed, the LTP preferentially catalyze transfers of exogenous phospholipids to the outer leaflet of membranes (33). The presence of 22:6n-3-phospholipids in both membrane leaflets could be important to observe a full 22:6n-3 inhibitory effect on TXA_2/PGH_2 receptors. Considering the hydrophobicity of TXA_2, this compound and its mimetics might bind to one of the receptor transmembrane domains (24).

The underlying mechanism of 22:6n-3-phospholipid inhibitory effect could involve changes in platelet membrane fluidity that are associated with alterations in platelet responsiveness to most aggregating agents (34-36). The fluidity parameters of enriched membranes by the mean of LTP, analyzed by one-way analysis of variance, were not significantly modified when compared to control membranes (Table 5). These results support the notion that 22:6n-3 acts specifically upon TXA_2/PGH_2 receptors through its direct interaction with the binding sites, based on structural similarities between 22:6n-3 and TXA_2/PGH_2 (37). It might also act by association with the hydrophobic domains on the receptors or on proteins of the receptor micro-environment which could in turn alter the receptor conformation.

In summary, a relatively large increase of the proportion of 22:6n-3 in diacyl-GPC of platelet membranes can induce an inhibition of TXA_2/PGH_2 receptor affinity to its ligands. Further studies are necessary to more precisely define the 22:6n-3 molecular species implicated in the TXA_2/PGH_2 receptor inhibition, their location in the membrane, and the nature of their interaction with TXA_2/PGH_2 receptors.

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